EGCG, a major polyphenol in green tea, protects human retinal pigment epithelium (ARPE-19) cells from viable blue light-induced disorders

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Purpose: Visible blue light-induced retinal pigment epithelium (RPE) cell death can be caused by a variety of cellular mechanisms that involve oxidative stress. The aim of the study was to determine whether epigallocatechin-3-gallate (EGCG) could prevent visible blue light-induced oxidative stress in human ARPE-19 cells. It may be useful in the prevention of early age related macular degeneration (AMD). **Methods:** Cultured human ARPE-19 cells were subjected to visible blue light in the presence and absence of EGCG. The viability of cells was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and morphological evaluation. The disorder of ARPE-19 cells was screen with the activities of superoxide dismutase (SOD). Apoptosis related protein, active caspase-3, was measured with western blot analysis. **Results:** Treatment with EGCG significantly decreased caspase-3 expression and increased SOD activity in human ARPE-19 cells. Moreover, the percentage of visible blue light induced-cellular death was significantly inhibited in the presence of ECGC cultured cell group compared with absence of EGCG group (* $p< 0.05$). **Conclusions:** The studies demonstrated that EGCG protects from the visible blue light induced-cellular disorder. EGCG mediated cytoprotection was likely mediated through the increase of antioxidant enzyme activity and the inhibition of active csapase-3 expression. The results of this study open new roads for the use of EGCG in the prevention of primary of early AMD where visible blue light plays a major role in disease pathogenesis.

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1. Introduction

Light-induced cell death can be caused by a variety of cellular mechanisms variety of cellular mechanisms that involve oxidative stress, reactive oxygen species (ROS) [1]. Light-induced ocular discords have been demonstrated by the results of human and animal studies. The cornea absorbs about 90% of UVB radiation and is most sensitive to UVB damage [2, 3]. However, the visible blue light particularly increases the risk of light damage to the retinal tissue [4-6] (Figure 1). Blue light exposure has a role in the progression of age-related macular degeneration (AMD) and can also contribute to retinitis pigmentosa, as also determined in the study based on human population [7]. In addition, it was demonstrated that blue light is risky to the retinal tissue of young rhesus monkeys with the continuous spectrum. Even though lower-power blue light is hazardous to retinal tissues as our previously study [8].

The retinal pigment epithelium (RPE) is a monolayer of pigmented cells forming a part of the blood-retina barrier that closely interacts with photoreceptors [9-11]. The RPE takes up and delivers nutrients, growth factors and ions and from blood to the photoreceptors. The RPE removes waste products of retinal metabolism from the subretinal space to the blood. Taken together, the RPE is essential for photoreceptor survival and, hence, for vision. Moreover, a layer of pigmented cells the RPE absorbs the light energy focused by the lens on the retina. Pathological studies indicate that primary of

early AMD where oxidative stress plays a major role in disease pathogenesis [12].

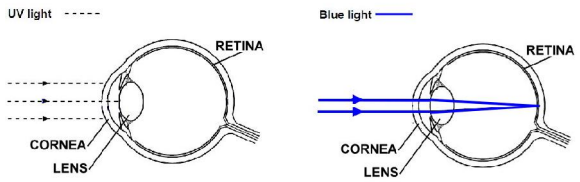

Fig. 1: Light-induced ocular discords
The cornea and lens absorb about 90% of UVB radiation and are most sensitive to UVB damage. However, the visible blue light is particularly increases the risk of disorder to the retina.

AMD is one of the most common causes of severe visual loss in the elderly population in the developed world [13, 14]. The neurodegenerative disease is characterized by loss of central vision as a result of cellular dysfunction and cell loss at the macula. The loss of vision results primarily from the progressive degeneration and death of RPE cells, which secondarily impairs the function of photoreceptors including rod and cone cells. Phenotypically, AMD can be classified into two main kinds: dry (atrophic or or non-neovascular) and wet (exudative or neovascular) forms and further subdivided into initial and late stages disorder. The early stage of dry AMD is asymptomatic, although pigment mottling, aggregation of intracellular lysosomal lipofuscin, and extracellular drusen deposits can be observed [15]. The late stage of dry AMD, also known as geographic atrophy, is characterized by discrete areas of RPE loss and impairment of the overlying retinal photoreceptor cells. In some individuals, as dry AMD deteriorates, blood vessel outgrowth may begin to induce by VEGF (vascular endothelial growth factor), and the condition can progress into wet AMD [16]. Sometimes within months, disorder to the macula occurs rapidly, occasioning in distorted or even lost central vision in wet AMD [17].

Epigallocatechin-3-gallate (EGCG), the most active major polyphenol of green tea, is mainly responsible for the green tea effects. It is important to note that EGCG is thought to act as an antioxidant in biological systems. Several pharmacological antioxidant properties of EGCG have been known such as free radical scavenging activity or reduction of lipid peroxidation due to various forms of radicals [18, 19] and inhibition of anthine oxidase activity [20]. Protective effects of EGCG on blue light-induced damage in human retinoblastoma Y79 cells also have been demonstrated as our previous study [8]. In this study, we proposed that EGCG protect human ARPE-19 cells from blue light effects. We investigated the beneficial effects of EGCG against blue light-induced human RPE cell death. It may be useful in the prevention of early AMD.

2. Material and Methods

Cell cultures

Adult human retinal pigment epithelial cells (ARPE-19) were purchased from Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were cultured in a humidified incubator at 37°C and 5% CO_2. These cells were maintained in in Dulbecco's MEM/Nut mix F-12 (1:1) medium (GibcoBRL, Invitrogen Life Technologies, CA) containing 10% heat-inactivated fetal bovine serum (GibcoBRL), 100 units/ml penicillin and 100 µg/ml streptomycin and 2 mM L-glutamine. The cells were grown to an appropriate density and used at passage 10-15. For most of the experiments, cells reaching 80-85% of confluence were starved and synchronized in serum-free DMEM for 24 h before they were subjected to further analysis.

Visible blue light irradiation

Spectrum distribution was analyzed using an UV-visible spectrophotometer (Ultrospec 3000, Pharmacia Biotech, Cambridge, UK) from 300 nm to 800 nm as our previous study [8]. The wavelength within the UV range was not detectable from the light source. The spectrum distribution of lighting began to increase at 300 nm and reached sustained maximum transmission at 450 nm of blue light.

EGCG treatment on cultured cells

EGCG was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Human ARPE-19 cells were treated with various concentrations of EGCG for 3 days. The medium with EGCG was replaced every 24 h for the duration of the experiment. Control cells received equal amounts of phosphate-buffered saline (PBS) (Sigma-Aldrich) in the medium. For the combination study, cells were treated with an optimal concentration of EGCG based on our following results for a total 3 days as the common recommended doses of these compounds.

Cell viability with MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) assay

To evaluate the effects of EGCG against visible blue light-induced cell death, cell viability was determined by the MTT assay. The cells were seeded on a 96-well tissue culture plate at 10,000 cells/cm^2 and incubated for 24 h prior to drug exposure. Briefly, PBS- or EGCG-pretreated ARPE-19 cells were exposed to visible blue light and incubated for an additional 24 h. After a brief wash

with medium, 0.5 mg/ml MTT in DMEM was used for the quantification of living metabolically active cells. After 24 h, the cells were incubated with MTT solution (0.1 mg/ml in PBS) for an additional 3 h at 37°C. The MTT solution was then aspirated off. To dissolve the formazan crystals formed in viable cells, 100 µl DMSO was added to each well. Absorbance was measured at 540 nm using a spectrophotometer. Cells cultured without blue light irradiation served as the blank controls. All groups were tested in triplicate for statistical evaluation. The cell viability was proportional to the absorbance measured.

Measurement of superoxide dismutase (SOD) levels

The SOD activity was determined by a superoxide dismutase assay kit provided by Cayman Chemical Co. (Michigan, USA). A homogenate was prepared in ice-cold 50 mM phosphate buffer (pH 7.4) containing a mammalian protease inhibitor cocktail and centrifuged at 10,000×g for 30 minutes at 4°C. Next, the supernatant was used to analyze the activities of SOD assay and evaluated in a spectrophotometer.

Cell fractionation and Western Blot

Samples were washed once with ice-cold PBS and then lysed with PBS containing 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1 µg/ml leupeptin, 10 µg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride for 10 min. After extract total protein with Total Protein Cell Lysis Buffer (Amresco Inc., OH, USA), crude extracts were subjected to centrifugation at 4°C. The supernatants were collected as cell lysates. All protein concentrations were determined by a protein assay (Bio-Rad laboratories, Richmond, California). Aliquots (50 µg) of cell lysates or nuclear extracts were separated electrophoresed on 8 or 12% SDS polyacrylamide gel and then transblotted onto the ImmobilonTM-P membrane. After being blocked with 10% skim milk in Tween-20/PBS, blots were incubated with active caspase-3 or β-actin antibodies and then incubated with HRP-conjugated secondary antibodies. The protein bands in the blots were detected using enhanced chemiluminescence kit (ECL; PerkinElmer Life Sciences, Inc. Boston).

Morphological evaluation

The cultured cells were fixed in 4% paraformaldehyde for 15 min, washed 3 times in PBS, and covered with cold 100% methanol for 10 minutes. Briefly, cells were stained with hematoxlin (Sigma-Aldrich). After 5 more rinses in PBS, samples were mounted and viewed on a Zeiss Axiophot microscope (Carl Zeiss).

Statistical analysis

The results are expressed as the mean ± SEM and were evaluated for significance. Statistical analysis was using a student's t-test. A p value of less than 0.05 or 0.01 was considered significant.

3. Results

3.1 Visible blue radiation-induced cell death

To ratify the cytotoxic effect of visible blue light irradiation, we exposed cultured ARPE19 cells to increasing doses of visible blue light exposure (15, 25, 35, 45, and 55 mW/cm^2). Cell viability was determined at 24 h after light irradiation. ARPE-19 cells suffered cell death after blue light exposure in our system. The decrease of cell viability was visible blue light-dose-dependent, resulting in 88.6, 75.2, 52.6, 42.7, and 37.2% of remaining survivals at 15, 25, 35, 45, and 55mW/cm^2 (Figure 2). Since 35mW/cm^2 of visible blue light irradiation caused adjacent a half decrease of cell viability (52.6%), the experimental dosage was used in the succeeding studies on the protective effect on light induced cell disorder.

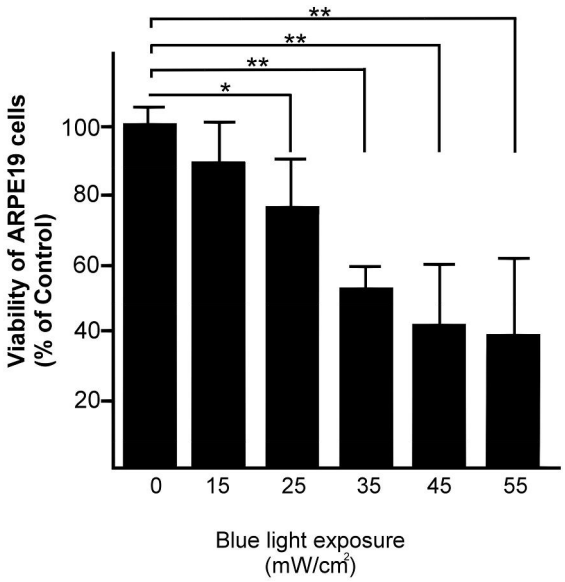

Fig. 2: Viability of ARPE-19 cell with blue light-induced disorder

Cell viability of ARPE-19 cells was decreased associated with dose-dependent of visible light. 35mW/cm^2 of visible blue light irradiation caused adjacent a half decrease of cell viability. * indicates $p< 0.05$ as compared to the blank control. ** indicates $p < 0.01$ as compared to the blank control.

Table 1 Antioxidant enzyme SOD activities

Antioxidant enzyme activities	Blank control	Blue light exposure	
		Vehicle	EGCG
SOD (OD values %)	100.0±6.2	43.3±8.2	86.2.0±6.2*

* indicates $p< 0.05$ as compared to the vehicle control following light exposure.

3.2 Increase of intracellular SOD activity with EGCG treatment

As SOD is the major cellular anti-ROS agent, we measured SOD activity following EGCG incubation. Treatment with visible blue light caused an obvious decrease (about 43%) in the total intracellular SOD activity in RPE cells, correspondingly, as compared to the blank control group. EGCG pretreatment significantly prevented a decrease in SOD activity, respectively, when compared with the blue light irradiation-group. The mean was significant difference at the $p<0.05$ level (Table 1). These data suggest that EGCG could trigger the cellular anti-oxidative system to protect ARPE-19 cells.

3.3 Decrease of cell death with EGCG treatment

To determine the protective effects of EGCG on ARPE19 cells, we first performed a cell viability assay. The MTT assay showed that cell viability of

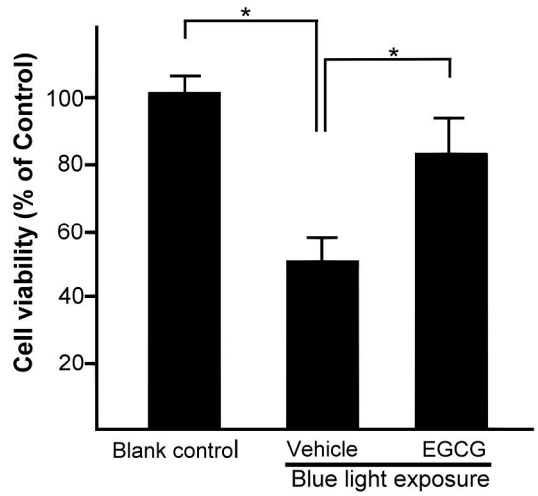

Fig. 3: Effects of EGCG on cell viability of ARPE-19 cells following blue light exposure
Cell viability of ARPE cells was increased with EGCG pretreatment. Data are expressed of three independent experiments in triplicate. * indicates $p< 0.05$.

ARPE-19 cells was decreased after blue light exposure. However, the decrease was reversed by the treatment of EGCG (Figure 3). The different in cell survival rate under blue light irradiation with of EGCG and without EGCG was significant ($p<0.05$). In addition, ARPE-19 cells exposure to blue light showed a significant increase in apoptosis as indicated by active caspase-3 using western blot analysis, correspondingly, as compared to the control group. However, the expression of active caspase-3 induced by blue light damage was inverted with EGCG pretreatment (Figure 4).

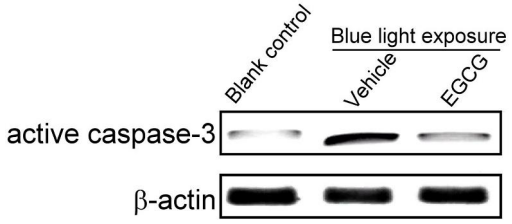

Fig. 4: Effects of EGCG on active caspas-3 in ARPE-19 cells following blue light exposure
The increase of active caspase-3 expression was attenuated with EGCG treatment. β-actin was used as a positive control

3. 4 Decrease of the cell number of cell death with chromatin condensation with EGCG treatment

The morphological evaluation was also performed to determine whether EGCG treatment can inhibit cell death induced by visible blue light. The number of the nuclei with characteristic condensed chromatin cells was very few in control cultured cells; whereas the number of undergoing cell death in ARPE-19 cells was significantly increased in blue light exposure-group. However, pretreatment of EGCG prior to light exposure led to a dramatic decrease in the numbers of cell death with chromatin condensation (Figure 5).

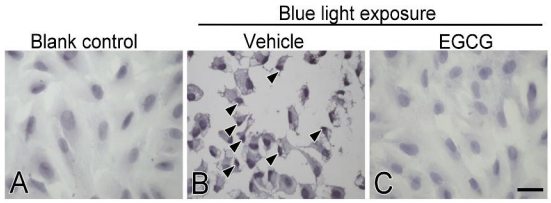

Fig. 5: Morphological evaluation of degeneration response by EGCG in ARPE-19 cells
The cytotoxic responses were measured by the morphological evaluation. The cell number of cell death with chromatin condensation (arrowheads) was significantly increased after blue light irradiation. Pretreatment with EGCG caused a significant decrease in the numbers of cell death. Scale bar: 20μm.

4. Discussion

The green tea extracts contain EGCG, (-)-epigallocatechin, (-)-epicatechin gallate, (-)-epicatechin, and catechin [21]. Among these components, EGCG is the most abundant and most active component in green tea effect. Various mechanisms have been demonstrated for the anticancer properties of EGCG, including protection from cellular oxidative stress, inhibition of angiogenesis, and regulation of signal transduction [22, 23]. Protective effects of EGCG on blue light-induced damage in human retinoblastoma Y79 cells also have been demonstrated as our previous study [8]. In the present study, we demonstrated that EGCG has a cytoprotective effect from blue light-induced disorders in the ARPE-19 cells that were likely mediated through the increase of antioxidant enzyme activity and the inhibition of active csapase-3 expression.

The retinal tissue is protected from ultraviolet light by the cornea and lens, which absorb ultraviolet light, but can be injured by visible blue light [24]. Previous study indicated that exposure to blue light induce retinal photoreceptor disorder as evidenced by various examinations, including the functional ERG assay, IHC, TUNEL, and TEM [25]. Moreover, cell culture studies indicated that blue light directly induces the production of ROS in mitochondria of RPE cells [26], potentially generated by ROS damage to mitochondrial DNA [27]. In the present study, we also found obvious decrease in the total intracellular SOD activity in RPE cells with visible blue light irradiation. As SOD is the major cellular anti-ROS agent, the decrease of intracellular SOD activity might lead to mitochondrial damage and cell dysfunction over time [28].

The visual deficiency in AMD is due to the degeneration of the RPE layer. Recently, the women's health initiative sight exam study on hormone treatment and AMD revealed that therapy with conjugated equine female steroid hormone may decrease the risk of soft drusen or wet AMD. Numerous other studies also suggest that there is a reduced risk of AMD with postmenopausal hormone replacement therapy. Epidemiologic data suggest that estrogen, one of the female steroid hormones, is protective against AMD, but that severity of AMD in women (as compared with men) increases in proportion to duration from onset of menopause [29, 30]. According to the results of these studies, the possibility that the cytoprotective functions of ARPE-19 cell from blue light-induced injury was mediated by estrogen receptors. Studies also indicated that estrogen receptor are found in a variety of human ocular tissues, such as retinal pigment epithelium, corneal epithelium , photoreceptors, retinal ganglion cells, retinal bipolar cells, lens epithelium, ciliary body epithelium, and iris stroma, demonstrated by RT-PCR assays, western blot assays and immune-cytochemistry [31]. In the cultured cells study, EGCG can affect chromatin modifications and the binding alteration of the transcription repressor complex with the ER promoter, resulting in ER reactivation [22]. Our studies also demonstrated that EGCG protect from the cell death of ARPE-19 cells induced by visible blue light. Taken together, it will be appropriate to conclude that the EGCG exerts its RPE cell damage-preventing action, although precise mechanism remains need to be address.

5. Conclusion

EGCG increases human RPE cells survival after exposure to visible blue light radiation. The studies demonstrated that EGCG protect from the cellular death induced by visible blue light. EGCG mediated cytoprotection was likely mediated through the increase of antioxidant enzyme activity and the inhibition of active csapase-3 expression. The results of this study may open new roads for the use of EGCG in the prevention of primary of early AMD where visible blue light plays a major role in the disease pathogenesis. EGCG may be appropriate to be further developed as a prophylactic health food for the prevention of retinal diseases. In addition, our results imply that green tea catechins can have a beneficial effect and can play an important role in the prevention of visible light-induced visual disorders.

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